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Slaving the cytochrome P-450 dependent monooxygenase system by periodically applied light pulses

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Abstract. The light-induced enhancement of 7-ethoxycoumarin-O-deethylase activity was measured in a reconstituted system consisting of the enzyme P-450 II B1 ($P\text{-}450_{PB-B}$) and the NADPH-cytochrome P-450 reductase. The phases of the catalytic cycle of $2 \cdot 10^{12}$ protein complexes were locked by periodic application of light pulses (0.1 s duration, 1.2–2.5 s repetition time, and 390–470 nm 0.27 Joule/nmol P-450). More than 80% of the active reconstituted enzyme complexes worked in phase if the repetition time (1.32 s) was slightly smaller than the catalytic cycle time of the free running enzyme (1.54 s). The percentage of synchronized enzyme complexes as a function of the repetition time is shown. It is shown that the lifetime of the product-enzyme complex is shortened by the light.

Key word: Enzyme activity

1. Introduction

A new type of enzyme activity enhancement by light has been described by Müller-Enoch and Gruler (1986) and Häberle et al. (1990). Absorption of light by the active group of the enzyme results in *(i)* the release of an inhibitor, as shown by the classical experiment of Warburg (1932) or *(ii)* in an enhancement of the turnover number as first shown by Müller-Enoch and Gruler (1986). In this paper we show that the light induced enhancement of the turnover number is due to a shortening of the catalytic cycle time. In addition, we show that nearly all catalytic active enzyme complexes ($2 \cdot 10^{12}$) can be synchronized by light flashes, if the appropriate repetition time of the light pulses is chosen (Häberle et al. 1990), so that up to 80% of the "molecular machines" work in phase.

Abbreviations: P-450, liver microsomal cytochrome P-450; PB, phenobarbital

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The heme containing cytochrome P-450 dependent monooxygenase system used in the experiments plays an important role in the oxidative metabolism of lipophilic substrates in eukaryotic and prokaryotic species (Guengerich 1987, Nebert and Gonzalez 1987). The overall process of the P-450-catalyzed oxidation can be described by a catalytic cycle (White and Coon 1980).

2. Materials and methods

The apparatus and the reconstituted system used were as follows. Liver P-450 II B1 and NADPH-cytochrome P-450 reductase were purified to electrophoretic homogeneity from PB-treated rats using procedures described by Guengerich et al. (1982). The purified P-450 II B1 enzyme was mixed with the NADPH-P-450 reductase in small test tubes (final volume 50 µl) by adding the P-450 enzyme to the reductase in a 1 : 2 molar ratio at high concentrations (7.5 and 15 µ*M*) as described by Müller-Enoch et al. (1984). The light induced enhancement of the 7-ethoxycoumarin O-deethylase activity was performed with the preformed P-450 : NADPH-reductase complex, diluted with 0.1 *M tris*-HCl-puffer (pH 7.6) containing 20% glycerol to yield final concentrations of P-450 and NADPH-P-450 reductase of 1.875 and 3.75 µ*M*, respectively. Aliquots of this complex (8 µl ≡ 15 pmol P-450) were added to a cuvette containing (in a total volume of 130 µl) 0.1 M *Tris*-chloride buffer (pH 7.6), 20% glycerol (v/v), 0.1 m*M* 7-ethoxycoumarin, 3.3 m*M* $MgCl_2$ and 0.115 m*M* NADPH.

At 30 °C the substrate (7-ethoxycoumarin) is transformed into the product (7-hydroxycoumarin) with a specific activity of 9.3 nmol product per min and per nmol of P-450. The product concentration was measured spectroscopically. The excitation wavelength was 365 ± 8 nm. The detection unit was oriented perpendicularly with an emission wavelength of 460 ± 10.5 nm. The second light source faced the first and was adjusted to the action spectrum of the catalytic reaction (Müller-Enoch and Gruler 1986) (390–460 nm). The irradiation time, as

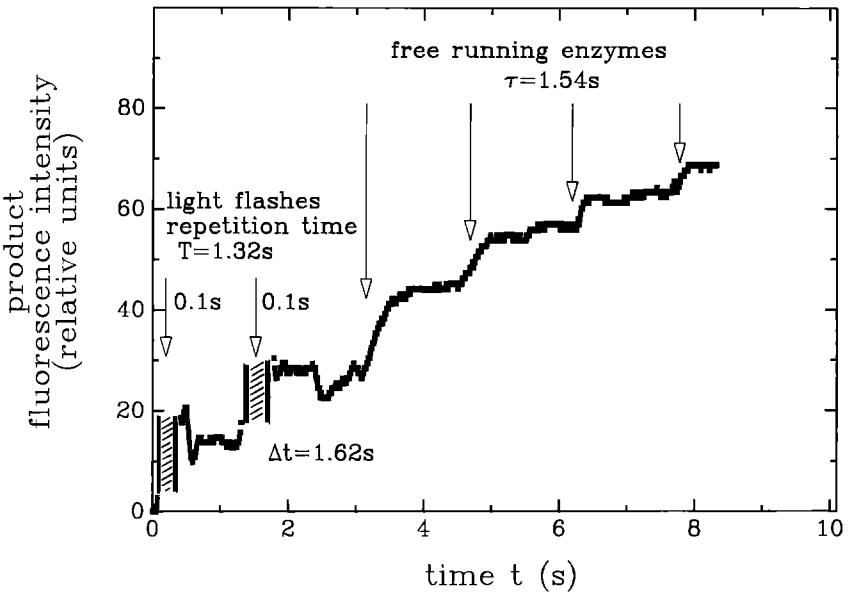

Fig. 1. Effect of periodically (1.32 s) applied short light pulses (0.1 s, 390–460 nm, 0.27 Joule/nmol P-450) on product formation for 7-ethoxycoumarin O-deethylase activity produced by a reconstituted P-450 II B1 : NADPH-P-450 reductase complex. The product concentration (7-hydroxycoumarin) in the test tube increases in a step-like manner before and after the last light flash. The arrows (above 2 s) represent the location of the step-like product formation in the case of coherent working enzyme complexes

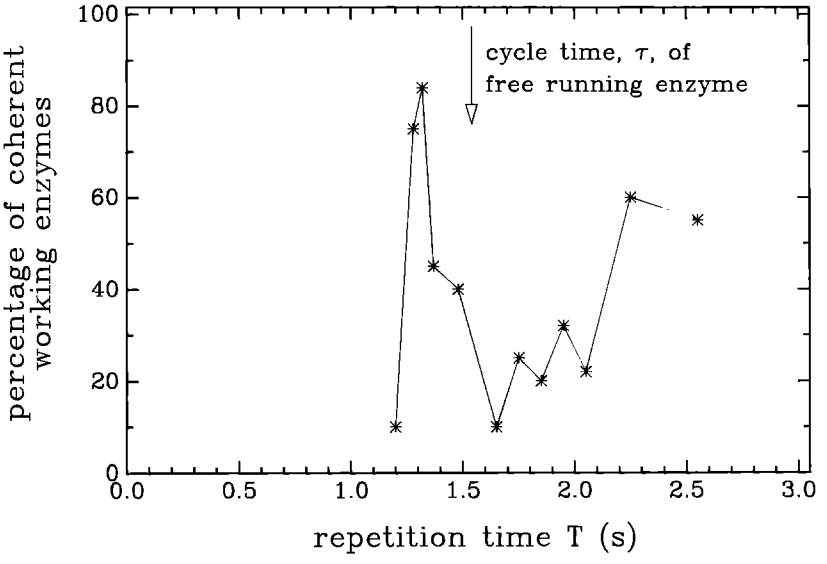

Fig. 2. The repetition time of the light flashes was altered between 1.2 and 2.5 s. The conditions for the reconstituted P-450 : reductase complex activity are given in the legend of Fig. 1. The percentage of coherent working enzyme complexes was obtained by evaluating two different parts of the step function: the extrapolated step height and the finite slope after the step. The mean value of the percentage of coherent working enzymes after application of 10 light flashes as a function of the repetition time is shown. The arrow indicates the catalytic cycle time of the free running enzyme reaction

well as the repetition time, could be varied over a wide range by switching a chopper controlled by a computer.

The absorption of light could lead to an increase in the temperature of the solution which would have the consequence of stimulating the enzyme activity. Under the conditions used we were not able to measure an increased temperature. This is in accordance with the estimated temperature increase when all the light is adsorbed in the cuvette. The light energy of one flash was 4 mJ (=40 mW · 0.1 s). The temperature in a solution of 130 µl could be increased by $7 \cdot 10^{-3}$ °C per flash. This value must be regarded as an upper limit since the mass of the cuvette as well as the thermal contact to a thermostat are neglected in the calculation. Thus, the light induced enzyme activity enhancement is not simply attributable to an increased temperature.

3. Coherent and incoherent enzyme activity

In general, all cycles of the working enzyme system in a test tube are independent of each other. For this situation, the product concentration increases linearly with time. Now let us assume that all the catalytic cycles have a fixed and constant phase relation. In this case the product concentration increases with time in a step function and not linearly as in the case of uncorrelated cycles. We achieved this result by triggering the cycles by an appropriate periodic irradiation of the reaction mixture as shown in Fig. 1 for the last two light flashes.

The cycle time or the characteristic time of the working enzyme is the time required for a single catalytic cycle and can be obtained by taking the inverse of the maximum enzyme activity under optimum conditions.

Müller-Enoch et al. (1984) obtained for P-450 II B1, with 7-ethoxycoumarin as substrate, a cycle time, τ, of 1.54 s ($=(39\ \mathrm{min}^{-1})$). This value can be used as a starting position for the repetition time of the light pulses. If the repetition time, T, is greater than or equal to the catalytic cycle time, τ, we expect that most of the enzymes will be working out-of-phase. After several flashes we found that a large fraction of the enzymes were not working in-phase (Fig. 2). The other extreme case is where the repetition time is very short compared with the cycle time. Under such conditions one would not expect to find a large fraction of enzymes working in phase (Fig. 2). The best repetition time for slaving the enzymes is just below the characteristic time, τ, of the free running enzyme as shown in Fig. 2.

In consequence, if there are in-phase working enzyme complexes in the test tube then the product concentration should increase in time as a step function after the last flash. This behaviour was actually observed as shown in Fig. 1. The repetition time between two steps is the cycle time of the free running reaction. We measured a time of 1.54 s. This corresponds to a turnover number of $39\ \mathrm{min}^{-1}$. Müller-Enoch et al. (1984) measured the maximum enzyme activity under optimum conditions and found, for the same enzyme and for the same substrate, a value of $39\ \mathrm{min}^{-1}$. The comparison of these two types of experiments shows that it is possible to measure the maximum catalytic activity of a given enzyme under conditions which are not optimum. The free, coherently working enzyme provides direct evidence for the turnover number of the investigated enzyme. This is a fundamental finding and we assume that the method can be applied to every enzymatic reaction.

In the experiment performed only 24% of the two enzymes (reductase and P-450 II B1) form a catalytically active complex. The catalytic cycle time can be determined under such non-optimal condition as shown above. In addition, one can determine a lower limit of the lifetime of the enzyme complex as 4 catalytic cycle times since after the last flash the coherence of the cycles still exists for a period of time, as can be seen in Fig. 1.

The light induced enhancement of the enzyme activity can only act in the intermediate states which are rate limiting for the catalytic reaction, and when the enzyme system is able to absorb light. Three of the eight steps of the enzyme reaction cycle are seriously considered as rate limiting steps (White and Coon 1980): *(i)* the transfer of the second electron, *(ii)* the generation of the "active oxygen", and *(iii)* the product dissociation. In a previous publication (Müller-Enoch and Gruler 1986) we showed that the enzyme complexes of the three rate limiting steps have absorption bands which are in accordance with the photochemical action spectrum of the reconstituted enzyme system used. The temporal behaviour of the product concentration after the last flash yields information concerning the rate limiting steps. Our experimental finding is that the lag time, Δt, between the last light flash and the next step in product concentration is about the cycle time of the free running enzyme. This fits well if product dissociation is an important rate limiting step. In the case of the assumption that the product dissociation is a less important rate limiting step (life time small compared with the life time of the other rate limiting steps) then the next rate limiting step is responsible for the effect, etc. To answer this question a new apparatus has to be built to allow the adsorption spectrum of all intermediate states to be measured as a function of time.

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